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# Specific degradation of CRABP-II via cIAP1-mediated ubiquitylation induced by hybrid molecules that crosslink cIAP1 and the target protein

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ABSTRACT

**Manipulation of protein stability with small molecules is a challenge in the field of drug discovery. Here we show that cellular retinoic acid binding protein-II (CRABP-II) can be specifically degraded by a novel compound, SNIPER-4, consisting of (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine methyl ester and all-trans retinoic acid that are ligands for cellular inhibitor of apoptosis protein 1 (cIAP1) and CRABP-II, respectively. Mechanistic analysis revealed that SNIPER-4 induces cIAP1-mediated ubiquitylation of CRABP-II, resulting in the proteasomal degradation. The protein knockdown strategy employing the structure of SNIPER-4 could be applicable to other target proteins.**


## 1. Introduction

The ubiquitin–proteasome system (UPS) plays a crucial role in selective degradation of proteins, which is involved in the regulation of cell cycle, proliferation, differentiation and cell death [1–3]. In the UPS, the target protein is poly-ubiquitylated by the sequential action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3), where E3 determines the specificity for the target protein in many cases. The poly-ubiquitylated proteins are degraded by proteasome. Inappropriate regulation of the UPS results in an accumulation or depletion of certain proteins, which results in a variety of diseases such as cancer, cachexia, neurodegenerative disorders and malformation [4–8]. To reduce a pathogenic protein in cells, it is useful to take advantage of the UPS since it is a highly specific system to target a certain protein for degradation, and the degradation occurs very quickly. However, few studies have been reported so far on the specific degradation of target proteins utilizing the UPS [9].

We previously reported that a class of small molecules, represented by (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine methyl ester (MeBS), destabilize cellular inhibitor of apoptosis protein 1 (cIAP1), a ubiquitin-ligase (E3) belonging to IAP (inhibitor of apoptosis protein) family [10–12], and sensitize cancer cells to apoptosis induced by anti-cancer drugs and death receptor ligation [13–16]. MeBS directly interacts with cIAP1 at its BIR3 domain and induces auto-ubiquitylation of cIAP1 depending on its RING domain, resulting in the proteasomal degradation of cIAP1. Structure–activity relationship study indicated that analogs with a carboxyl-ester reduce the amount of cIAP1 even though the methyl group is substituted to other residues, and other modifications of MeBS seriously affected the activity [13]. Based on these observations, we hypothesized that the methyl group can be substituted to a ligand for a target protein without ablating the ubiquitin ligase activity of cIAP1. The hybrid molecule consisting of MeBS and the ligand would be able to cross-link cIAP1 and the target protein, to induce ubiquitylation and proteasomal degradation of the target protein. As a proof-of-concept study, we designed and synthesized a series of hybrid compounds consisting of MeBS and all-trans retinoic acid (ATRA), and found that they

*Abbreviations:* cIAP1, cellular inhibitor of apoptosis protein 1; MeBS, (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine methyl ester; ATRA, all-trans retinoic acid; CRABP, cellular retinoic acid binding protein

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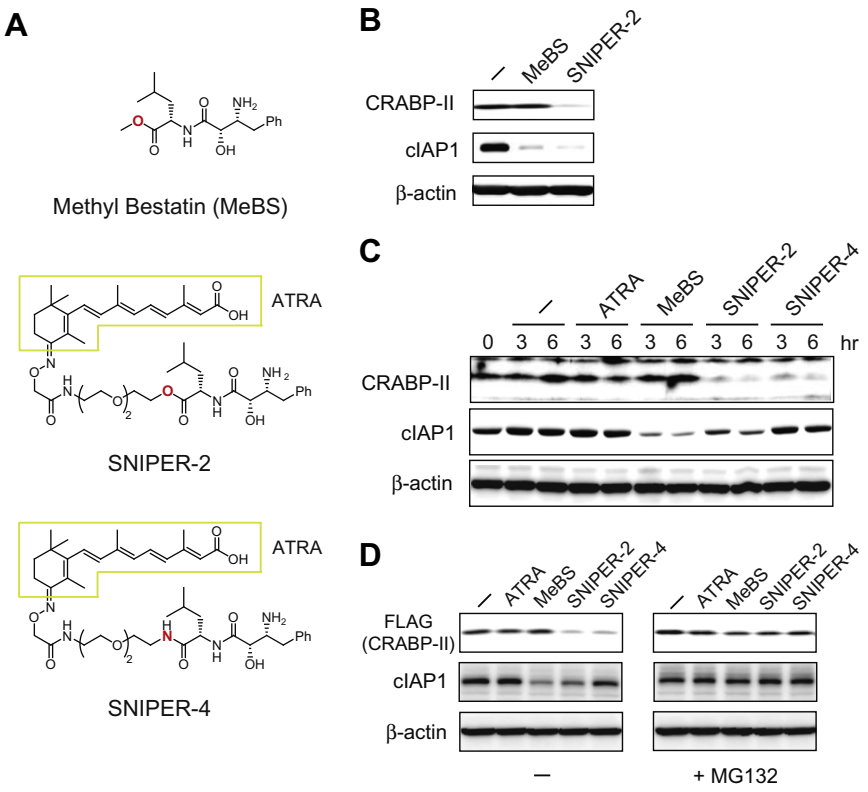


**Fig. 1.** Structure and protein knockdown activity of SNIPERs. (A) Structures of MeBS (top), SNIPER-2 (middle) and SNIPER-4 (bottom). (B) Reduction of endogenous CRABP-II and cIAP1 by SNIPER-2. (C) SNIPER-4 reduces endogenous CRABP-II but not cIAP1. (D) Inhibition of the SNIPER-mediated protein knockdown by a proteasome inhibitor, MG132. Human primary fibroblasts (B), HT1080 (C) or HT1080 cells expressing FLAG-CRABP-II (D) were incubated with 10 μM of indicated compounds for 6 h. Cells were pre-treated with 10 μM of MG132 for 30 min in (D). Shown are immunoblots of cell lysates stained with indicated antibodies.

induce the proteasomal degradation of CRABP-II, an ATRA-binding protein [17]. Thus, selective degradation of target protein (protein knockdown) can be attained by the hybrid molecules that cross-link the target protein and cIAP1.

The hybrid molecule we have developed previously [17], which we named SNIPER (Specific and Non-genetic IAP-dependent Protein ERaser)-2 in this study, however, has two major downsides that would be unfavorable for protein knockdown: (1) SNIPER-2 causes a simultaneous degradation of cIAP1 with the target protein CRABP-II, which may make the protein knockdown unsustainable, and (2) SNIPER-2 contains an ester-bond which is vulnerable to hydrolysis. To overcome these issues, we here developed SNIPER-4, in which the ester-bond is substituted to amide-bond, and found that the SNIPER-4 induces specific and sustained degradation of CRABP-II without inducing cIAP1 degradation. We also investigated biochemical mechanisms by which SNIPERs target CRABP-II for degradation, and demonstrate that SNIPERs induce the ubiquitylation of CRABP-II, which is mediated by cIAP1 but not by XIAP.

## 2. Materials and methods

### 2.1. Reagents and plasmids

MeBS was kindly provided by Nippon Kayaku Co. Ltd. (Tokyo, Japan). SNIPER-2 was synthesized as described previously [17]. The synthesis and structural analysis of SNIPER-4 would be reported elsewhere (Itoh et al., manuscript in preparation). cDNA encoding human CRABP-II were amplified by PCR from JHH-5 cDNA library and cloned into a p3xFLAG-CMV-10 expression vector (SIGMA). The correct DNA sequence was confirmed. The following reagents were purchased from indicated supplier: M2 anti-FLAG mouse monoclonal antibody, M2 anti-FLAG agarose-conjugated antibody, mouse anti-β-actin antibody (SIGMA); Fugene HD, anti-HA rat monoclonal antibody (Roche); CRABP-II antibody (Abcam); anti-human cIAP1 goat polyclonal antibody (R&D systems); anti-human XIAP mouse monoclonal antibody (MBL); Lipofectamine RNAi MAX transfection reagent, Stealth Select RNAi (Invitrogen). The target sequences for cIAP1 and XIAP RNAi were as follows: cIAP1-#1 (5′-TCTAGAGCAGTTGAAGACATCTCTT-3′); cIAP1-#2 (5′-GGAAATGCTGCGGCCAACATCTTCA-3′); XIAP-#1 (5′-ACACTGGCACGAGCAGGGTTTCTTT-3′); XIAP-#2 (5′-CCAGAATGGTCAGTACAAAGTTGAA-3′).

### 2.2. Cell culture, transfection and treatment with compounds

Human fibrosarcoma HT1080 were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 μg/ml of kanamycin. Transient transfections were carried out using Fugene HD according to the manufacturer's instructions. siRNA transfections were carried out by reverse transfection method with Lipofectamine RNAi MAX according to the manufacturer's instructions. In brief, 60 pmol siRNA and 3 μl of Lipofectamine RNAi MAX were incubated in 200 μl Opti-MEM medium for 20 min at room temperature in 12-well plates, and then 1 ml of cell suspension ($1.5 \times 10^5$ cells) was added to the siRNA–RNAi MAX complex. HT1080 cells constitutively expressing FLAG-CRABP-II were generated and maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 μg/ml of kanamycin and 500 μg/ml of Geneticin (G418). The cells were treated with 10 μM SNIPERs or vehicle (DMSO) for 6 h unless otherwise indicated, and lysed in

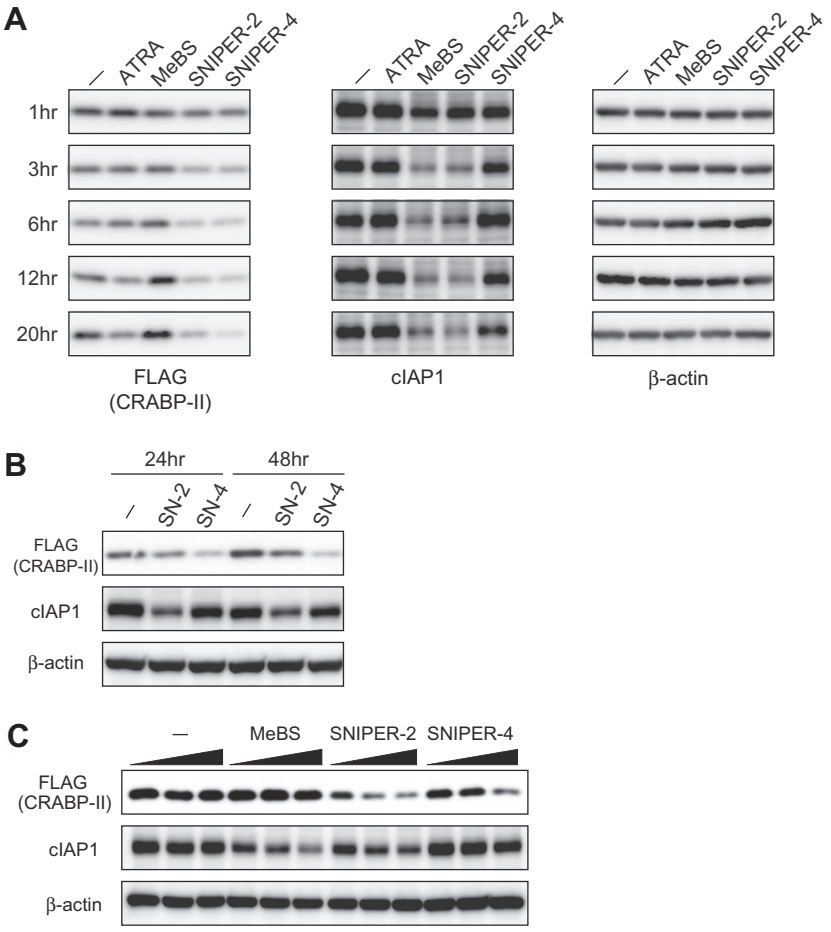


**Fig. 2.** (A and B) Time-course and (C) dose-dependent response of CRABP-II degradation induced by SNIPERs. HT1080 cells constitutively expressing FLAG-CRABP-II were incubated with 10 μM compounds for indicated times (A and B), or 0.3, 1 or 3 μM of compounds for 6 h (C, from the left of black triangle). Cell lysates were Western blotted with indicated antibodies.

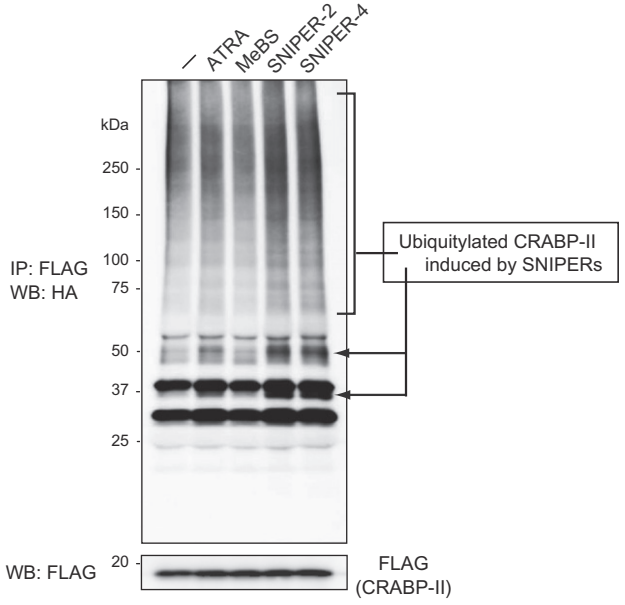


**Fig. 3.** Ubiquitylation of CRABP-II induced by SNIPERs. HT1080 cells expressing FLAG-CRABP-II and HA–ubiquitin were pre-treated with MG132 for 30 min, and then incubated with 10 μM of compounds for 1 h. Cells were lysed and FLAG-CRABP-II was immunoprecipitated with anti-FLAG antibody. The ubiquitylated CRABP-II was detected with anti-HA antibody.

lysis buffer (1% SDS, 0.1 M Tris–HCl (pH 7.0), 10% glycerol) and boiled for 10 min. Protein concentration was measured by BCA method (Pierce) and the equal amount of protein lysate was separated by SDS–PAGE, transferred to Hybond-P (GE Healthcare) membrane and Western blotted using appropriate antibody. Protein signals were detected using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo scientific) or ECL™ Western Blotting Detection Reagents (GE Healthcare).

### 2.3. Ubiquitylation of CRABP-II

HA–ubiquitin was transiently transfected in HT1080 cells constitutively expressing FLAG-CRABP-II. Cells were lysed in lysis buffer and boiled, and the lysates were diluted 10 times with 0.1 M Tris–HCl. CRABP-II was immunoprecipitated with anti-FLAG agarose-conjugated beads. The immunoprecipitates were extensively washed with diluted lysis buffer, and analyzed by Western blotting using anti-HA antibody.

## 3. Results and discussion

The structures of MeBS (top), SNIPER-2 (middle) and SNIPER-4 (bottom) are shown in Fig. 1A. These SNIPERs are hybrid molecules consisting of bestatin moiety and ATRA connected by an ester-bond (SNIPER-2) or an amide-bond (SNIPER-4) spacer. SNIPER-2 induces the degradation of endogenous CRABP-II and

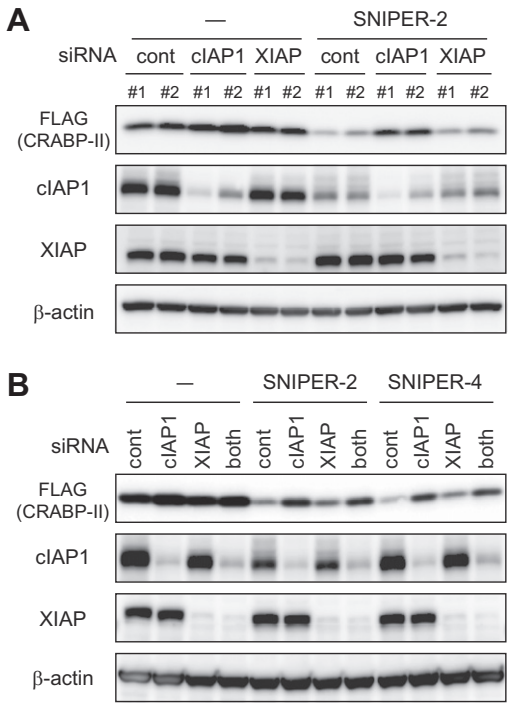


**Fig. 4.** Silencing of cIAP1 attenuates the SNIPER-dependent CRABP-II protein degradation. In HT1080 cells expressing FLAG-CRABP-II, the endogenous cIAP1 and XIAP were knocked down by two different siRNAs (#1 or #2) against each protein for 48 h (A). cIAP1, XIAP or both were knocked down by siRNAs (cIAP1-#1, XIAP-#2) (B). Cells were incubated with 10 μM compounds for 6 h. Shown are immunoblots of cell lysates stained with indicated antibodies.

cIAP1 in human primary fibroblasts as we reported previously (Fig. 1B) [17]. To develop a novel SNIPER that reduces CRABP-II but not cIAP1, we designed SNIPER-4 in which the ester-bond is substituted to amide-bond, because our previous study indicated that bestatin methyl amide could bind to cIAP1 without reducing it [13]. Fig. 1C shows that the SNIPER-4 reduced the level of CRABP-II but not cIAP1 in HT1080 cells as we speculated. SNIPER-2 reduced both the CRABP-II and cIAP1, while MeBS reduced cIAP1 specifically. Likewise, in HT1080 cells expressing FLAG-tagged CRABP-II, SNIPER-4 reduced FLAG-CRABP-II but not cIAP1. The combined use of MeBS and ATRA did not induce the degradation of CRABP-II (Supplementary Fig. 1). In addition, the reduction of CRABP-II by SNIPER-4 and SNIPER-2, and that of cIAP1 by MeBS and SNIPER-2, were all abrogated by a proteasome inhibitor, MG132 (Fig. 1D). These results indicate that SNIPER-2 induces proteasomal degradation of CRABP-II and cIAP1 while SNIPER-4 degrades CRABP-II specifically, and that the linking MeBS and ATRA in one molecule is required for the CRABP-II degradation.

Then, we evaluated the effect of SNIPERs on the target protein degradation, examining the reaction time-course and the treatment dose. As shown in Fig. 2A, SNIPER-2 and -4 reduce the CRABP-II at 1 h, then kept suppressing the CRABP-II expression over 20 h. On a longer time scale, the expression levels of CRABP-II at 24 or 48 h were lower in the cells treated with SNIPER-4 than with SNIPER-2 (Fig. 2B). This may be due to the maintenance of cIAP1 level in the SNIPER-4-treated cells (Fig. 2A and B), and/or the chemical stability of SNIPER-4 compared with SNIPER-2 since ester-bond is more easily hydrolyzed than amide-bond. We also tested the dose–response of SNIPERs on CRABP-II degradation. The CRABP-II was effectively reduced by over 3 μM and subtly affected by 0.3 μM or 1 μM of SNIPER-4 (Fig. 2C and Supplementary Fig. 2).

Next, we examined whether the SNIPERs induce the ubiquitylation of CRABP-II as we assumed. Lysates from the cells expressing FLAG-tagged CRABP-II and HA-tagged ubiquitin were immunoprecipitated with anti-FLAG (CRABP-II) and the immunoprecipitates were analyzed by Western blot with anti-HA (ubiquitin) to detect the ubiquitylated CRABP-II. Smear protein bands that migrate slowly in the gels increased by SNIPER-2 and SNIPER-4 (Fig. 3), which indicates the poly-ubiquitylation of CRABP-II. We further examined whether cIAP1 is the ubiquitin ligase responsible for the degradation of CRABP-II in the SNIPER-treated cells. Silencing cIAP1 expression by siRNAs significantly suppressed the SNIPER-mediated CRBAP-II degradation, whereas silencing XIAP, a close family member of cIAP1, did not (Fig. 4A and B). Expression of cIAP2, another close family member, is hardly detected in HT1080 cells (data not shown). These results indicate that cIAP1 is the primary ubiquitin ligase for CRABP-II.

Fig. 5 shows the schema of the protein knockdown by MeBS, SNIPER-2 and SNIPER-4. MeBS interacts with BIR3 domain of cIAP1 to induce auto-ubiquitylation mediated by its RING domain for proteasomal degradation (Fig. 5, top). SNIPER-2 with ester-bond cross-links cIAP1 and CRABP-II, and induces ubiquitylation of both proteins, resulting in the degradation of cIAP1 and CRABP-II by proteasome (Fig. 5, middle). SNIPER-4 with amide-bond also cross-links cIAP1 and CRABP-II, but it specifically induces proteasomal degradation of CRABP-II but not cIAP1 (Fig. 5, bottom). Thus, the amide-type SNIPER-4 would be more useful than the ester-type SNIPER-2 for protein knockdown in terms of the specificity and prolonging the duration of the degradation process.

The reason why SNIPER-4 does not degrade cIAP1 is not fully understood. We previously reported that bestatin-methyl ester (MeBS) induced proteasomal degradation of cIAP1, whereas bestatin-methyl amide (BE-04) did not (13). Therefore, we hypothesize that BE-04 and SNIPER-4 with amide-bond would not induce auto-ubiquitylation of cIAP1, or if any it is not enough for efficient proteasomal degradation. Probably, the robustness and/or kinetics of the auto-ubiquitylation would be much lower than that induced by MeBS and SNIPER-2 with ester-bond, and therefore, de-ubiquitylation of cIAP1 quickly occurs to prevent degradation.

To study physiological functions of certain proteins, genetic knockdown by RNA interference or genetic knockout by gene targeting was commonly applied for suppressing the expression. Comparing with such genetic methods, the protein knockdown by SNIPERs has several advantages: (i) SNIPERs are small molecules and easily delivered into cells, which is especially advantageous for medical application in future. (ii) The degradation of the target protein begins soon after the addition of SNIPERs, and therefore the protein knockdown is attained in several hours. We suppose that the protein knockdown by SNIPERs could be a complementary technology to RNA interference, and if combined it may be possible to downregulate a target protein more rapidly and robustly. This is especially the case for a long-lived protein that is insufficiently downregulated by RNA interference alone.

The protein knockdown by SNIPERs depends on cIAP1-mediated ubiquitylation of the target protein. The cIAP1 is ubiquitously expressed in a variety of tissues and cells [18], indicating that the protein knockdown by SNIPERs would be attained in most tissues and cells expressing cIAP1. Since cIAP1 is involved in NFκB signaling in some cells, the effect of SNIPERs on normal cell function should be carefully investigated. Structurally, SNIPERs could target other proteins for degradation if ATRA is substituted to ligands for other target proteins. We propose the amide-type SNIPER could be applicable to downregulate pathogenic proteins for therapeutic purposes.

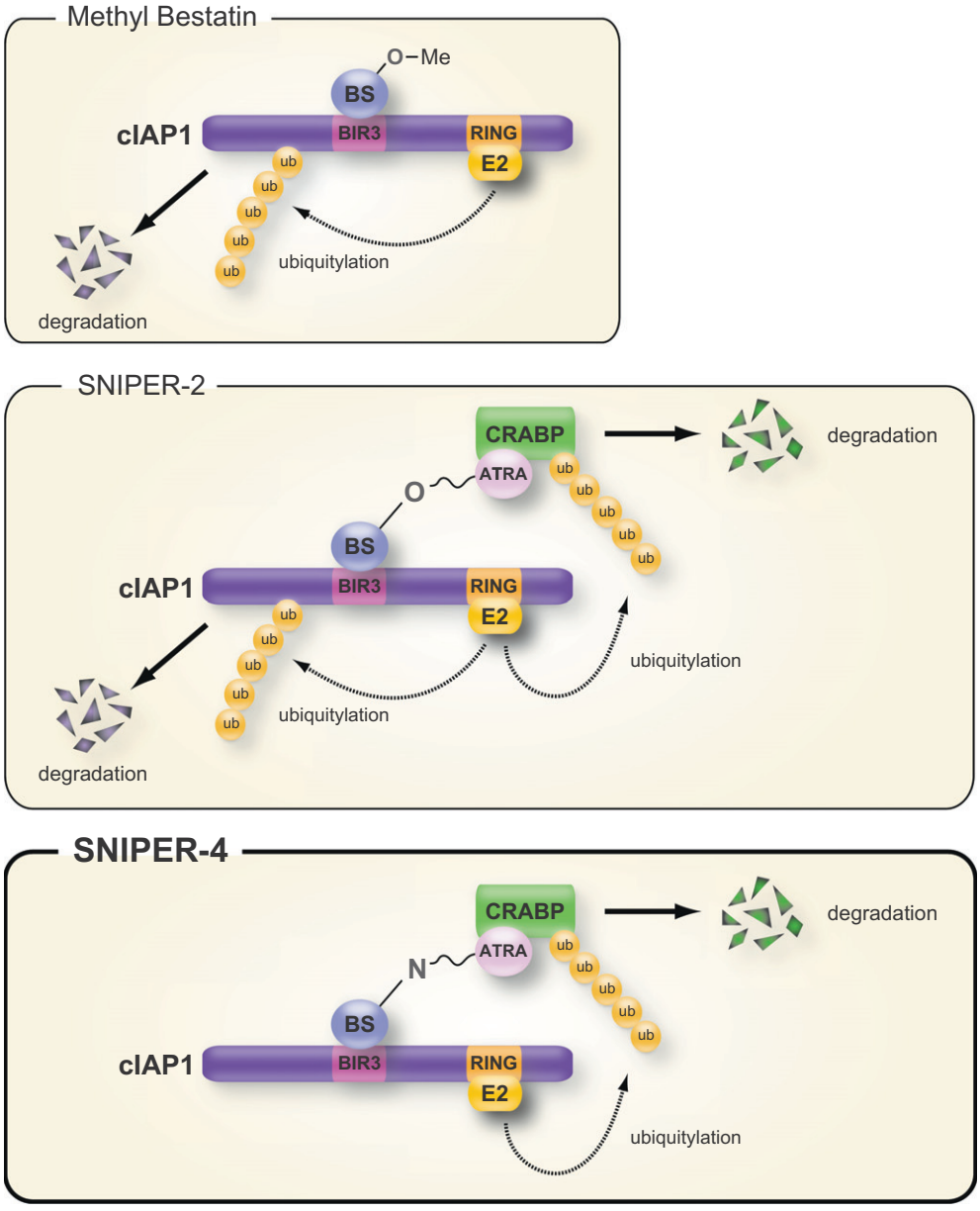


**Fig. 5.** Scheme of the protein knockdown by MeBS (top), SNIPER-2 (middle) and SNIPER-4 (bottom). See text for the explanation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.03.019.

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